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Reductions in Calcium Uptake Induced in Rat Brain Synaptosomes by Ionizing Radiation

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Gamma irradiation (^{60}Co) reduced KCl-stimulated voltage-dependent $^{45}Ca^{2+}$ uptake in whole-brain, cortical, and striatal synaptosomes. The time course (3, 10, 30, and 60 s) of calcium uptake by irradiated (3 Gy) and nonirradiated synaptosomes, as well as the effect of KCl (15–65 m*M*), was measured in whole-brain synaptosomes. The fastest and highest rate of depolarization-dependent calcium uptake occurred at 3 s with 65 m*M* KCl. Irradiation reduced calcium uptake at all incubation times and KCl concentrations. Bay K 8644 enhancement of KCl-stimulated calcium influx was also reduced by radiation exposure. Nimodipine binding to dihydropyridine (DHP) L-type calcium channel receptors was not altered following radiation exposure. These results demonstrate an inhibitory effect of ionizing radiation on the voltage-sensitive calcium channels in rat brain synaptosomes that are not mediated by DHP receptors.

INTRODUCTION

Although the central nervous system (CNS) is generally considered to be relatively resistant to the direct effects of ionizing radiation, exposure to ionizing radiation can have a complex effect on the CNS that is dependent on the dose and time elapsed after radiation exposure (*1–6*). The biochemical basis for radiation-induced behavioral/physiological changes mediated by the CNS is unknown.

Calcium plays several important roles in various electrophysiological and neurochemical processes, and it is generally accepted that extracellular calcium influx can be regulated by calcium channel agonists (i.e., cardiostimulatory drugs), or can be blocked by calcium channel antagonists (*7*). These antagonists are structurally heterogenous and include verapamil, diltiazem, D-600, and the dihydropyridine (DHP) compounds, such as nimodipine and nifedipine (*8*). These drugs exhibit a common locus of action on voltage-sensitive calcium channels (VSCC) (*9, 10*). Several types of VSCC have been identified, of which L, N, and T are most important. The DHP antagonists mentioned above are specific for the L-type VSCC, which is characterized by large conductances of long duration (*7*).

Calcium channel binding sites for DHP compounds have been located in the brain and heart using nimodipine and nifedipine (*11–13*). Nimodipine binding provides a molecular probe by which the kinetics of calcium receptors may be evaluated (*12*). A slight structural modification of DHP calcium antagonists yields Bay K 8644 [methyl-1,4-dihydro-2,6-dimethyl-3-nitro-4-2(trifluoromethylphenyl) pyridine-5-carboxylate], which confers agonist activity for calcium influx in the brain (*13–18*) and smooth and cardiac muscle (*19, 20*). Interestingly, a direct effect of Bay K 8644 on synaptosomal calcium entry in rat brain has not been observed (*21–23*).

The present experiments indicate a reduction in calcium uptake after irradiation in whole-brain, cortical, and striatal synaptosomes. This study characterizes the effect of ionizing radiation on voltage-dependent calcium influx by measuring KCl-stimulated $^{45}Ca^{2+}$ uptake into rat whole-brain, cortical, and striatal synaptosomes. Additionally, calcium channel receptor binding studies were done to determine whether the reductions were due to any alterations in DHP L-type receptors.

MATERIALS AND METHODS

Materials. Bay K 8644 and nimodipine were gifts from Miles Laboratories, Inc. (West Haven, CT) and were dissolved in dimethylsulfoxide (DMSO). Due to the light sensitivity of these compounds, all experiments were done using foil-wrapped test tubes. $CaCl_2$ and [^{3}H]nimodipine were purchased from New England Nuclear (DuPont, Boston, MA), and all other chemicals and reagents used were of the highest analytical grade and were obtained from Sigma (St. Louis, MO).

Experimental animals. Male Sprague-Dawley rats weighing 200–300 g (Charles River Breeding Laboratories, Kingston, NY) were quarantined on arrival and screened for evidence of disease by representative serology and histopathology sampling before being released from quarantine. The rats were housed individually in polycarbonate Micro Isolator cages (Lab Products, Maywood, NJ) on autoclaved hardwood contact bedding (Beta Chip Northeastern Products Corp, Warrenburg, NY) and provided with commerical rodent chow and acidified water (pH = 2.5) *ad libitum*. Animal holding rooms were kept at 21 ± 1°C with 50 ± 10% relative humidity on a 12-h light:dark cycle with no twilight.

Tissue preparation and calcium uptake. Animals were killed by decapitation, and the brain was removed from the skull and placed on ice. Whole brain, cortex, and striatum were dissected using the method of Glowinski and Iversen (*24*), and the synaptosomal pellet (P2) from these regions was, or pellets were, prepared by a modification of the method of Gray and

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Whittaker (25). The final pellet was resuspended in ice-cold incubation medium containing 136 m*M* NaCl, 5 m*M* KCl, 1.3 m*M* $MgCl_2$, 0.12 m*M* $CaCl_2$, 10 m*M* glucose, and 20 m*M* Tris base, with the pH adjusted to 7.65 with 1.0 *M* maleic acid to provide a concentration range of approximately 4–6 mg protein/ml and divided into two equal portions. One portion was irradiated with γ photons while the other was used as a sham-irradiated control.

Aliquots (480 μl) of the synaptosomal preparation were transferred into test tubes and incubated for 14 min at 30°C in a Dubnoff metabolic shaker in the presence or absence of various concentrations of Bay K 8644 as detailed below. The Bay K 8644 or DMSO vehicle was added as a 20-μl volume to make a final incubation volume of 500 μl. Following the incubation period, 500 μl of depolarizing or nondepolarizing solution containing $^{45}Ca^{2+}$ (3 μCi) was added. The depolarizing solution was the same composition as that of the incubation medium, except that a portion of the NaCl was replaced by an osmotically equivalent amount of KCl to provide final KCl concentrations of 15, 30, or 65 m*M*. Calcium uptake was terminated by addition of 5 ml of an ice-cold buffer containing 3 m*M* EGTA (ethylene glycol bis(β-aminoethyl ether) *N, N'*-tetraacetic acid), 10 m*M* glucose, and 20 m*M* Tris base, with the pH adjusted to 7.65 with 1.0 *M* maleic acid. Each sample was then immediately filtered under vacuum through a presoaked Whatman GF/B filter. Excess and loosely bound $^{45}Ca^{2+}$ was eliminated with two 5-ml washes of ice-cold incubation medium. The filters were then placed in scintillation vials and radioactivity was determined by liquid scintillation spectrometry.

Net uptake of $^{45}Ca^{2+}$ into synaptosomes was calculated by subtracting the uptake in the absence of depolarization (5 m*M* KCl) from the uptake in the presence of depolarization (15, 30, or 65 m*M*). This value will be referred to as *k* (potassium-induced change) and represents net KCl-induced calcium uptake (*26, 27*).

[^{3}H]Nimodipine binding assays. The binding assay was done based on the methods reported by Skattebol and Triggle (*28*). Whole brain, cortex, and striatum were obtained and isolated as described above and pooled according to protein needs for significant binding determined in preliminary experiments (data not shown). The P2 pellet was prepared as described above and resuspended in 50 m*M* Tris base buffer (pH 7.4) and divided into two equal portions, one of which was irradiated with γ photons, while the other was used as a sham-irradiated control. Synaptosomes were irradiated to maintain procedural continuity with the calcium uptake studies. Following irradiation, the synaptosomes were homogenized for a second time (glass/glass) in 3 ml buffer to form membrane fragments, which were aliquoted (200 μl: 100 μg/sample, striata; 400 μg/sample, whole brain; 400 μg/sample, cortex) into black snap-cap polypropylene test tubes. Half of these tubes contained 5 μ*M* unlabeled nimodipine (25 μl/sample) for determination of nonspecific binding. A preliminary displacement experiment indicated that the apparent K_d for this system was approximately 1 n*M* (data not shown).

Based on preliminary experiments, maximal binding was obtained in 20 min (data not shown). [^{3}H]Nimodipine (NEN, 123.3 Ci/m*M*, 25 μl/sample) was added to the aliquoted samples (in duplicate, final volume 565 μl) over a concentration range of 50 p*M* to 0.8 n*M* for 1 h at 4°C. The binding experiment was then terminated using a 24-place Brandel Cell Harvester (Gaithersburg, MD), and the membrane fragments were collected on Whatman [illegible] filters, followed by 3 × 3-ml washes with 50 m*M* Tris base buffer. The filters were then transferred to scintillation vials. After determining radioactivity by liquid scintillation spectrometry, the data were converted to fmol and expressed as fmol/mg protein.

Irradiation procedures. It has been reported that no significant oxygen uptake occurs in whole-brain homogenates *in vitro* after irradiation, which suggests that the brain synaptosomes will not consume enough oxygen to affect the results[1] (*29*). To maintain the maximum viability of the synaptosomes, they were placed in glass test tubes in a Plexiglas ice bath and were exposed to radiation using a ^{60}Co source at a dose rate of 1 Gy/min (for the 1-Gy dose only), 10 Gy/min (for the 3-, 5-, and 10-Gy doses), or 20 Gy/min (for the 30-Gy dose). Dosimetry was performed using a 0.05-cm^3 tissue-equivalent ion chamber. The ion chamber was placed in a glass test tube inside the Plexiglas ice bath during dosimetry measurements.

Miscellaneous methods. Protein content was determined by the method of Lowry *et al.* (*30*), using bovine serum albumin as the standard. Statistical analysis was performed using Student's *t* test. Multiple comparisons with a control were done by analysis of variance (RS1; BBN Software Products Corp., Cambridge, MA) and Dunnett's test (*31*). Data were identified as significant if $P < 0.05$.

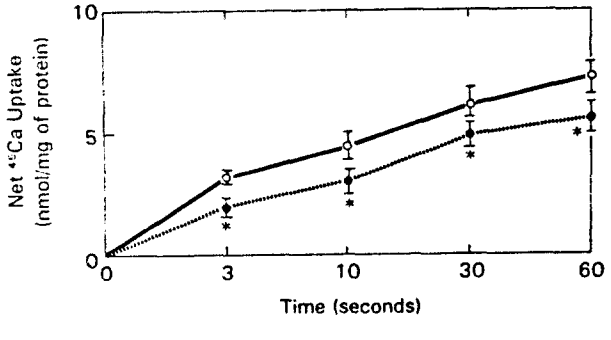

FIG. 1. The time course of 65 m*M* KCl-stimulated calcium uptake in nonirradiated (○) and irradiated (●) rat whole-brain synaptosomes. Points and bars represent means ± SEM values from three separate experiments, each using triplicate samples. A significant effect of ionizing radiation is indicated as follows: $^*P < 0.05$.

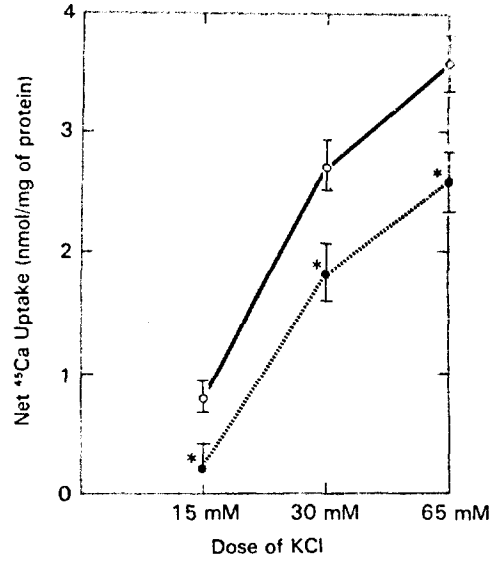

FIG. 2. Effects of 15, 30, and 65 m*M* KCl-stimulated calcium uptake in nonirradiated (○) and irradiated (●) rat whole-brain synaptosomes. Points and bars represent means ± SEM values from three separate experiments, each using triplicate samples. A significant effect of ionizing radiation is indicated as follows: $^*P < 0.05$.

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TABLE I
Effect of γ Radiation on 65 m*M* KCl-Stimulated $^{45}Ca^{2+}$ Uptake by Rat Whole-Brain, Cortex, and Striatal Synaptosomes

Dose of radiation (Gy)	Dose rate (Gy/min)	Net uptake of $^{45}Ca^{2+}$ (nmol/mg protein)[a]		
		Whole brain	Cortex	Striatum
Sham	Sham	3.4 ± 0.05	3.3 ± 0.05	3.5 ± 0.10
1	1	3.0 ± 0.10*	3.3 ± 0.10	3.3 ± 0.10
3	10	2.7 ± 0.05*	3.0 ± 0.10	3.0 ± 0.10*
5	10	2.4 ± 0.10*	2.8 ± 0.10*	2.8 ± 0.10*
10	10	2.0 ± 0.05*	2.5 ± 0.05*	2.5 ± 0.05*
30	20	1.7 ± 0.05*	2.2 ± 0.05*	2.4 ± 0.05*

[a] Values are means ± SEM of three separate experiments, each using triplicate samples.
* Significantly different from sham value: $P < 0.05$.

RESULTS

The time course (3, 10, 30, and 60 s) as well as the dose effect of KCl (15–65 m*M*) (Figs. 1 and 2) of calcium uptake was reduced following exposure of the whole brain to 3 Gy of γ photons. The fastest rate of depolarization-dependent calcium uptake occurred at 3 s (Fig. 1). The KCl dose–response curve (Fig. 2) showed that 65 m*M* KCl stimulated calcium uptake most. In the above two studies, irradiation reduced calcium uptake at all incubation times and KCl concentrations. Based on the above data, a time point of 3 s and KCl dose of 65 m*M* were selected for investigation of the radiation dose–response curve (Table I).

Gamma irradiation (1–30 Gy) reduced 65 m*M* KCl-stimulated calcium uptake after 3 s in whole-brain, cortical, and striatal synaptosomes. A comparison between regions showed a significant interaction term. Whole-brain synaptosomes were clearly more sensitive to irradiation than those of the cortex or striatum.

Gamma irradiation (1–30 Gy) also reduced 15 m*M* KCl-stimulated calcium uptake in whole-brain, cortical, and striatal synaptosomes after 3 s (Table II). The enhancement of 15 m*M* KCl-stimulated calcium uptake by Bay K 8644 following γ photon exposure was reduced by irradiation (3 Gy) (Fig. 3). Because the differences in uptake for the whole-brain, cortical, and striatal synaptosomes are very similar, only the whole-brain results are shown in Fig. 3. However, the same concentrations of Bay K 8644 did not enhance 30 or 65 m*M* KCl-stimulated calcium uptake (data not shown).

The effects described above indicate that γ irradiation will reduce KCl-stimulated calcium uptake. Several hypotheses can be advanced to explain these data. The initial hypothesis we investigated concerned calcium channel recognition receptor binding. The data showing that Bay K 8644-stimulated calcium uptake is reduced by γ irradiation justifies the selection of nimodipine, the antagonist for the calcium L-channel, as the appropriate ligand with which to perform binding studies.

Data obtained from whole-brain, cortical, and striatal membranes demonstrate that the specific binding of [^{3}H]-nimodipine is unaffected by γ irradiation at 10 Gy (data not shown). These data indicate that the significance of the reduction in KCl and/or Bay K 8644-stimulated calcium uptake is not due to alteration of the L-type calcium channel recognition receptor.

TABLE II
Effect of γ Radiation on 15 m*M* KCl-Stimulated $^{45}Ca^{2+}$ Uptake by Rat Whole-Brain, Cortex, and Striatal Synaptosomes

Dose of radiation (Gy)	Dose rate (Gy/min)	Net uptake of $^{45}Ca^{2+}$ (nmol/mg proteins)[a]		
		Whole brain	Cortex	Striatum
Sham	Sham	2.6 ± 0.05	2.7 ± 0.10	2.7 ± 0.10
1	1	2.4 ± 0.10	2.6 ± 0.10	2.6 ± 0.10
3	10	2.1 ± 0.05*	2.2 ± 0.05*	2.2 ± 0.10*
10	10	1.3 ± 0.10*	1.5 ± 0.15*	1.5 ± 0.15*
30	20	1.3 ± 0.15*	1.1 ± 0.20*	1.1 ± 0.20*

[a] Values are means ± SEM of three separated experiments, each using triplicate samples.
* Significantly different from sham value: $P < 0.05$.

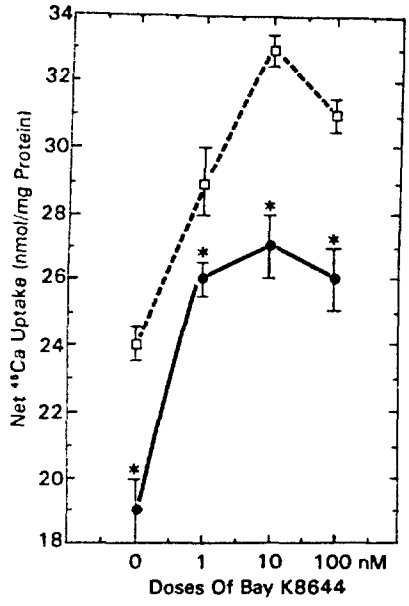

FIG. 3. Effect of γ radiation on Bay K 8644-induced 15 m*M* KCl-stimulated calcium uptake in nonirradiated (□) and irradiated (●) rat whole-brain synaptosomes. Points and bars represent means ± SEM values from three separate experiments, each using triplicate samples. A significant effect of ionizing radiation is indicated as follows: $^*P < 0.05$.

DISCUSSION

This study indicates that ionizing radiation decreases KCl-stimulated calcium uptake in the whole brain, cortex, and striatum. These results support electrophysiological studies suggesting reduced neuroexcitability after radiation exposure (*32, 33*) and contradict reports of radiation-induced increases in excitability (*34*). To our knowledge, this is the first report indicating reduction in KCl-stimulated calcium uptake following irradiation.

Bay K 8644 significantly increased the net voltage-dependent entry of calcium stimulated by 15 m*M* KCl in rat whole brain, cortex, and striatum. Our findings are consistent with previous reports about the potentiating action of Bay K 8644 on synaptosomal calcium channel activity, or KCl-stimulated norepinephrine, acetylcholine, and serotonin release from rat brain slices (*14–18*). The effectiveness of Bay K 8644 only at submaximal KCl concentrations agrees with the study by Woodward *et al.* (*35*). These findings are also consistent with reports suggesting that DHP agonists do not potentiate calcium entry into brain synaptosomes at high levels of KCl depolarization[2] (*35*).

The negative data from [^{3}H]nimodipine binding to whole brain, cortex, and striatum indicate that radiation-induced inhibition of Bay K 8644-enhanced KCl-stimulated or KCl-stimulated calcium uptake is not a result of an altered L-type calcium channel recognition receptor. Apparently, radiation does not decrease calcium uptake by a DHP-sensitive mechanism. Further work is in progress to determine the role of N and T calcium channels in mediating radiation-induced decreased calcium uptake.

In conclusion, the present data indicate that ionizing radiation significantly reduces KCl-stimulated calcium uptake.

At the present time we have no data to explain the differences in sensitivity to irradiation of whole brain, cortex, and striatum. Additionally, it is not clear how ionizing radiation causes this dose-dependent decrease in calcium uptake. Preliminary experiments with inositol trisphosphate,[3] prostaglandins (*36*), and phorbol esters (*37*) suggest an impairment in protein kinase C activity that is linked to the opening or closing of ion channels (*38*). Further work is in progress to compare protein kinase C activity of irradiated and nonirradiated synaptosomes, and we are studying G protein receptor binding to determine whether radiation-induced decreases in calcium uptake are due to impairment of protein kinase C, or alterations in G protein receptors, or both.

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